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Pyrosequencing-An Alternative to Traditional Sanger Sequencing

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Abstract: Problem statement: Pyrosequencing has the potential to rapidly and reliably sequence DNA taking advantages over traditional Sanger di-deoxy sequencing approach. **Approach:** A comprehensive review of the literature on the principles, applications, challenges and prospects of pyrosequencing was performed. **Results:** Pyrosequencing was a DNA sequencing technology based on the sequencing-by-synthesis principle. It employs a series of four enzymes to accurately detect nucleic acid sequences during the synthesis. Pyrosequencing had the potential advantages of accuracy, flexibility, parallel processing and could be easily automated. The technique dispenses with the need for labeled primers, labeled nucleotides and gel-electrophoresis. Pyrosequencing had opened up new possibilities for performing sequence-based DNA analysis. The method had been proven highly suitable for single nucleotide polymorphism analysis and sequencing of short stretches of DNA. Pyrosequencing had been successful for both confirmatory sequencing and de novo sequencing. By increasing the read length to higher scores and by shortening the sequence reaction time per base calling, pyrosequencing may take over many broad areas of DNA sequencing applications as the trend was directed to analysis of fewer amounts of specimens and large-scale settings, with higher throughput and lower cost. **Conclusion/Recommendations:** The Competitiveness of pyrosequencing with other sequencing methods can be improved in future.

Key words: Pyrosequencing, Sanger sequencing, alternative, advantage

INTRODUCTION

Genome sequencing has provided us with powerful insights into the genetic make-up of the microbial world and has spearheaded a host of revolutionary technologies, such as microarrays and proteomics that have transformed the field of microbiological research. Sequence determination is most commonly performed using di-deoxy chain termination technology (Ronaghi, 2001). The chain termination sequencing method, also known as Sanger sequencing, was developed by Frederick Sanger and colleagues (Sanger *et al*., 1977), has been the most widely used sequencing method since its advent in 1977 and still is in use after more than 29 years. Despite all the advantages, there are limitations in this method, which could be complemented with other techniques (Gharizadeh *et al*., 2007). Recently, pyrosequencing has emerged as a new sequencing methodology (Ronaghi, 2001).

Limitations of Sanger Sequencing Method: The Sanger sequencing method (Sanger *et al*., 1977) has been the workhorse technology for DNA sequencing since it's invent. Though Sanger method is still considered by the research community as the gold standard for sequencing, it has several limitations such as- (1) A great limitation of the Sanger sequencing method for larger sequence output is the need for gels or polymers used as sieving separation media for the fluorescently labeled DNA fragments. (2) Relatively low number of samples could be analyzed in parallel. (3) Total automation of the sample preparation methods is difficult. (4) DNA fragments need to be cloned into bacteria for larger sequences. (5) High cost of sequencing. (6) Sequencing errors.6. Level of sensitivity (generally estimated at 10-20%) insufficient for detection of clinically relevant low-level mutant alleles or organisms. (7) *cis* or *trans* orientation of heterozygous positions may be difficult to resolve during data analysis. (8) Not readily scalable to achieve a throughput capable of efficiently analyzing complex diploid genomes at low cost. (9) de novo genome assembly is difficult (Ansorge, 2009; Hall, 2007).

Alternatives of Sanger Sequencing: Many research groups around the world have made effort to develop alternative principles of DNA sequencing. Three methods that hold great promise are sequencing by

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hybridization (Bains and Smith, 1988; Drmanac *et al*., 1989; Khrapko *et al*., 1989; Southern, 1989) parallel signature sequencing based on ligation and cleavage (Brenner *et al*., 2000) and pyrosequencing (Ronaghi *et al*., 1996; Ronaghi *et al*., 1998).

Pyrosequencing: Pyrosequencing technology is a novel DNA sequencing technology, developed at the Royal Institute of Technology (KTH) and is the first alternative to the conventional Sanger method for de novo DNA sequencing. This method relies on the luminometric detection of pyrophosphate that is released during primer-directed DNA polymerase catalyzed nucleotide incorporation. It is suited for DNA sequencing of up to one hundred bases and it offers a number of unique advantages (Gharizadeh, 2003). This technique is a widely applicable, alternative approach for the detailed characterization of nucleic acids. Pyrosequencing has potential advantages of accuracy, flexibility, parallel processing and can be easily automated. Furthermore, the technique avoids the need for labeled primers, labeled nucleotides and gel-electrophoresis. Pyrosequencing has been successful for both confirmatory sequencing and de novo sequencing (Ronaghi, 2000).

Principles of Pyrosequencing: Pyrosequencing technique is based on sequencing-by-synthesis principle (Hyman, 1988; Melamede, 1985) and on the detection of released Pyrophosphate (PPi) during DNA synthesis (Ronaghi, 2001). It employs a series of four enzymes to accurately detect nucleic acid sequences during the synthesis. In Pyrosequencing (Nyren and Skarpnack, 2001) the sequencing primer is hybridized to a single-stranded DNA biotin-labeled template and mixed with the enzymes; DNA polymerase, ATP sulfurylase, luciferase and apyrase and the substrates adenosine 5' phosphosulfate (APS) and luciferin (Gharizadeh *et al*., 2007).

Cycles of four deoxynucleotide triphosphates (dNTPs) are separately added to the reaction mixture iteratively. The cascade starts with a nucleic acid polymerization reaction in which inorganic PPi is released as a result of nucleotide incorporation by polymerase. Each nucleotide incorporation event is followed by release of inorganic Pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Subsequently the released PPi is quantitatively converted to ATP by ATP sulfurylase in the presence of APS. The generated ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin, producing visible light in amounts that are proportional to the amount of ATPs. The light in the luciferase-catalyzed reaction with a maximum of 560 nanometer wavelength is then detected by a photon detection device such as A Charge Coupled Device (CCD) camera or photomultiplier. Apyrase is a nucleotide-degrading enzyme, which continuously degrades ATP and non-incorporated dNTPs in the reaction mixture. There is a certain time interval (usually 65 sec) between each nucleotide dispensation to allow complete degradation. For this reason, dNTP addition is performed one at a time (Gharizadeh *et al*., 2007). Because the added nucleotide is known, the sequence of the template can be determined (Ronaghi, 2001). A schematic representation of pyrosequencing is shown in Fig. 1.

The generated light is observed as a peak signal in the pyrogram (corresponding to electropherogram in dideoxy sequencing) proportional to the number of nucleotides incorporated (a triple dGTP incorporation generates a triple higher peak) (Gharizadeh *et al*., 2007). During this synthesis process, the DNA strand is extended by complementary nucleotides and the DNA sequence is demonstrated by the pyrogram on a screen.

The slope of the ascending curve in a pyrogram displays the activities of DNA polymerase and ATP sulfurylase, the height of the signal shows the activity of luciferase and the slope of the descending curve demonstrates the nucleotide degradation (Gharizadeh, 2003). Base-callings are performed with integrated software, which has many features for related SNP and sequencing analysis (Gharizadeh *et al*., 2007). The overall reaction from polymerization to light detection takes place within 3-4 sec at room temperature (Ronaghi, 2001). ATP sulfurylase converts PPi to ATP in approximately 1.5 sec and the generation of light by luciferase takes place in less than 0.2 sec (Nyren and Lundin, 1985). Standard pyrosequencing uses the Klenow fragment of Escherichia coli DNA Pol I (Benkovic and Cameron, 1995).

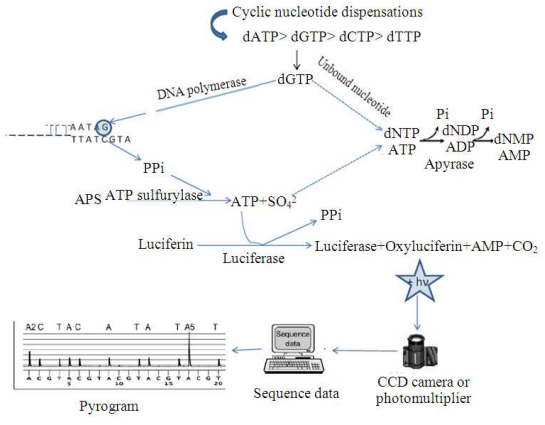

Fig. 1: Schematic representation of pyrosequencing

The ATP sulfurylase used in pyrosequencing is a recombinant version from the yeast Saccharomyces cerevisiae (Karamohamed *et al*., 1999) and the luciferase is from the American firefly Photinus pyralis (Ronaghi, 1999). The apyrase is from Solanum tuberosum (Pimpernel variety) (Espinosa *et al*., 2003; Nourizad *et al*., 2003).

Advantages of pyrosequencing: Pyrosequencing has emerged as an alternative method of sequencing. Although it has read-length limitations compared with di-deoxy sequencing, it is a fast method with real-time read-out that is highly suitable for sequencing short stretches of DNA (Gharizadeh *et al*., 2007).

Pyrosequencing employs co-operativity of several enzymes to monitor DNA synthesis. Parameters such as stability, fidelity, specificity, sensitivity, KM and kcat are mandatory for the optimal performance of the enzymes used in the sequencing reaction. The kinetics of the enzymes can be studied in realtime (Gharizadeh *et al*., 2003c).

Unlike Sanger sequencing, which lays a reading gap of roughly 20-30 bases from the sequencing primer, pyrosequencing can generate sequence signals immediately downstream of the primer. As sequencing starts with the first base next to the annealed primer, making primer design becomes more flexible in this method. Sample and single-strand DNA preparation process is also relatively rapid (about 15 min), while sample preparation takes approximately 4 h for Sanger sequencing (60 min for PCR cleanup, 3-4 h for cyclic amplification and 15 m for dye cleanup). The reagent costs are considerably lower for sequencing short stretches of DNA compared to currently available methods (Gharizadeh *et al*., 2007).

The pyrosequencing technology has many unique advantages over other DNA sequencing technologies. One advantage is that the order of nucleotide dispensation can be easily programmed and alterations in the pyrogram pattern reveal mutations, deletions and insertions. Moreover, this technique is carried out in real-time, as nucleotide incorporations and base callings can be observed continuously for each sample. In addition, the Pyrosequencing method can be automated for large-scale screenings (Gharizadeh *et al*., 2003c).

Industrialization of pyrosequencing: The availability of an automated system for liquid-phase pyrosequencing (PSQ 96 system, http://www.pyrosequencing. com) has allowed the technique to be adapted for high-throughput analyses (Ronaghi, 2001). Pyrosequencing is now being applied in microfluidic format commercially by 454 Life Sciences Corporation (Branford, CT, USA). The microfluidic pyroseqeuncing has been integrated with emulsion PCR and DNA sequencing assembly software. The new platform has the ca pacity to sequence up to 300,000 samples and generate up to 20-40 million bases at an accuracy of 99% per 4 h sequencing run (Gharizadeh *et al*., 2007).

Application of pyrosequencing: Pyrosequencing has opened up new possibilities for performing sequence-based DNA analysis (Ronaghi, 2001). Pyrosequencing is well suited for *de novo* sequencing and resequencing (Ronaghi, 2001). Currently, pyrosequencing method is broadly being used in many applications such as Single Nucleotide Polymorphism (SNP) genotyping (Ahmadian *et al*., 2000a; Nordstrom *et al*., 2000; Milan *et al*., 2000), identification of bacteria (Gharizadeh, 2003; Grahn *et al*., 2003; Jonasson *et al*., 2002), fungal (Gharizadeh *et al*., 2005; Trama *et al*., 2005) and viral typing (Gharizadeh *et al*., 2001; 2003; 2005; Adelson *et al*., 2005). Moreover, the method has demonstrated the ability to determine difficult secondary structures (Ronaghi, 2001) and perform mutation detection (Ahmadian *et al*., 2000b; Garcia *et al*., 2000), DNA methylation analysis (Neve *et al*., 2002; Uhlmann *et al*., 2002), multiplex sequencing (Gharizadeh *et al*., 2003a; 2006) (Gharizadeh *et al*., 2003b; Gharizadeh *et al*., 2006), tag sequencing of cDNA library (Nordstrom *et al*., 2001) and clone checking (Nourizad *et al*., 2003). Another highly significant application is whole genome sequencing (Margulies *et al*., 2005). Some of the potential applications of pyrosequencing have been described in Table 1.

Table 1: A collection of microbial applications using pyrosequencing

Organism	Application	Target	Reference
Eubacteria	Profiling and Identification	In stomachs of Mongolian without gerbils with or Helicobacter pylori	(Sun *et al*., 2003)
Mixed bacteria	Identification	In DNA-contaminated PCR amplifications of 16S DNA variable VR1 and VR3 regions	(Grahn *et al*., 2003)
General bacteria	Classification, Identification and subtyping	Analysis of 16S rDNA fragments	(Jonasson *et al*., 2002)
Lactobacilli	Identification	DNA VRs within colonies collected from normal vaginal fluid	(Tarnberg *et al*., 2002)
Helicobacter pylori	Profiling, Identification and subtyping Identification	NudA protein 16S DNA VR1 and VR3	(Lundin *et al*., 2003) (Sun *et al*., 2003; Monstein *et al*., 2001)
Listeria Monocytogenes	Grouping	Single Nucleotide Polymorphisms (SNP) in the inlB gene	(Unnerstad *et al*., 2001)
Papillomavirus	Typing	Human Papilloma Virus (HPV)	(Gharizadeh *et al*., 2001)
Human Immunodeficiency Virus (HIV)	Monitoring	Resistance to HIV type 1 Protease Inhibitors (PI)	(O'Meara *et al*., 2001)
Viruses	Quantitative	Estimation of viral fitness	(Lahser *et al*., 2003)

Challenges of pyrosequencing: Pyrosequencing was earlier limited to sequencing of short stretches of DNA, due to the inhibition of apyrase. The natural dATP was a substrate for luciferase, resulting in false sequence signals dATP was substituted by dATP-α-S (Ronaghi *et al*., 1996). By introducing the dATP- α -S Sp isomer, substantial longer reads were achieved. This improvement had a major impact on pyrosequencing read length and allowed sequencing of up to one hundred bases (Gharizadeh *et al*., 2002) and opened up avenues for numerous applications (Gharizadeh *et al*., 2007).

Homopolymer Ts (more than 3-4) are a challenge in Pyrosequencing. Homopolymer string (mainly homopolymeric T) regions can influence synchronized extension and synthesis of the DNA strand causing non-uniform sequence peak heights, affecting the read-length and possibly causing sequence errors. By employing Sequenase (Gharizadeh *et al*., 2004), an exonuclease deficient T7 DNA polymerase, the poly-T homopolymer string reads were significantly improved by generation of significantly more synchronized sequence and uniform signal peaks after homopolymeric T regions (Gharizadeh *et al*., 2007).

An important factor in pyrosequencing is primer design for PCR and sequencing. Sequencing primers should be checked for self-looping, primer-dimer (primer-primer hybridizations) and cross-hybridization (when more than one sequencing primer is used). (Gharizadeh *et al*., 2007).

An inherent problem with the described method is de novo sequencing of polymorphic regions in heterozygous DNA material (Ronaghi, 2001).

Another inherent problem is the difficulty in determining the number of incorporated nucleotides in homopolymeric regions, due to the nonlinear light response following incorporation of more than 5-6 identical nucleotides (Ronaghi, 2001).

Prospects of pyrosequencing: Future applications require more robust and efficient DNA sequencing techniques for sequence determination. The Pyrosequencing method has already shown evidence of high accuracy in DNA sequencing and analysis of polymorphic DNA fragments in many clinical and research settings. It is a relatively straightforward and user-friendly method possessing unique methodological characteristics and this technique is currently being used in multidisciplinary fields in academic, clinical and industrial settings. By increasing the read length to higher scores and by shortening the sequence reaction time per base calling, pyrosequencing may take over many broad areas of DNA sequencing applications as the trend is directed to analysis of fewer amounts of specimens and large-scale settings, with higher throughput and lower cost (Gharizadeh *et al*., 2007).

Pyrosequencing has shown excellent accuracy in analysis of polymorphic DNA fragments. This technology has also been used for quantification of allelic frequency in populations. Pyrosequencing will have a large impact in that area because a large number of samples can be pooled in one pyrosequencing reaction. A high throughput version of this technology can potentially be used for resequencing of genomes. Pyrosequencing technology is relatively new and there lies ample room for versatile developments in both chemistry and instrumentation. This technology is already time-and cost-competitive as compared to the most conventional sequencing methods. Work is underway to further improve the chemistry, to measure the sequencing efficiency at elevated temperatures and to run the reaction in miniaturized formats (Ronaghi, 2001).

CONCLUSION

Pyrosequencing method is the first alternative to the conventional Sanger di-deoxy method for de novo DNA sequencing. There are still opportunities for further breakthroughs in this area as only very few research groups have focused on this method. In future, pyrosequencing technology is expected to achieve longer read length, to reduce the sequencing time frame, to decrease the sample quantity and to make further improvements in automation.

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